ICE inhibitor YVADcmk is a potent therapeutic agent against *in vivo* liver apoptosis

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In liver, apoptosis is a physiological process involved in the clearance of injured cells and in homeostatic control [1]. However, in patients with viral fulminant hepatitis or with nonacute liver diseases [2], dramatic liver failure or secondary cirrhosis results from the death of hepatocytes, which express the cell-surface receptor Fas, by apoptosis. To date, treatment of fulminant hepatitis relies mainly on orthotopic liver transplantation, which is limited by immunological complications and graft availability. Unravelling the molecular mechanisms that underlie acute liver failure could allow the design of an appropriate therapy. Ligand-bound Fas and tumour necrosis factor α (TNF-α) induce hepatic apoptosis in mice [3–6]. In various cell types, Fas- or TNF-α-induced apoptosis is blocked by viral proteins (such as p35 and CrmA) as well as by a decoy peptide (YVADcmk) [7–11], suggesting that these mechanisms of apoptosis involve ICE (interleukin-1 β converting enzyme)-like proteases. Here, we report that, *in vivo*, pre-treatment of mice with YVADcmk protects them from the lethal effect of anti-Fas antibody and from liver failure induced by injection of TNF-α. Remarkably, YVADcmk administration is also highly effective in rescuing mice that have been pretreated with anti-Fas antibody from rapid death, despite extensive hepatic apoptosis. This dramatic curative effect could be of clinical benefit for the treatment of viral and inflammatory liver diseases.

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Results and discussion

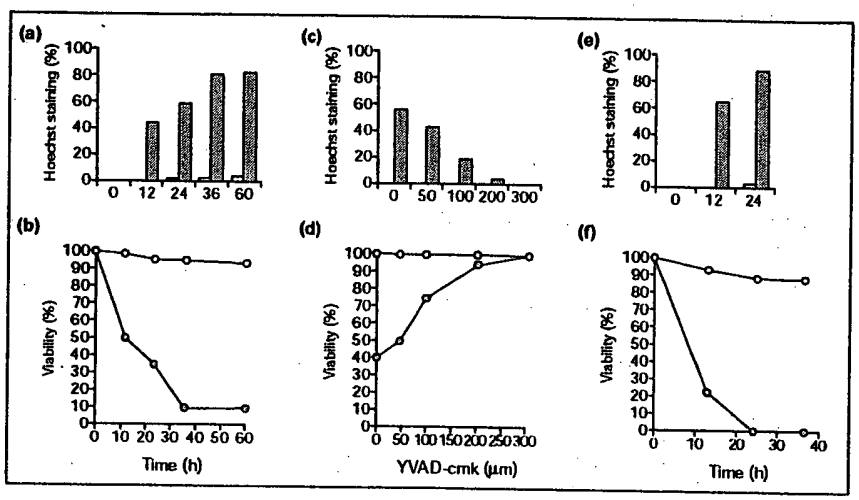
ICE-like proteases, mammalian homologs of the nematode cell death protein Ced-3, have apoptotic properties when overexpressed in various cell lines [12–19]. *In vitro*, the inhibition of these proteases by certain viral proteins, as well as by the YVADcmk peptide [7], results in protection against TNFα- and Fas-mediated apoptosis [8–11,20,21].

Primary cultured mouse hepatocytes undergo apoptosis after incubation with anti-Fas antibody in combination with cycloheximide (CHX) [22,23]. To investigate the role of ICE-like proteases in Fas-mediated apoptosis, primary hepatocytes were incubated with YVADcmk and then treated with anti-Fas antibody supplemented with CHX. In the absence of YVADcmk, 90 % of the cells had undergone apoptosis after 36 hours of incubation with anti-Fas antibody, whereas more than 90 % of the cells were resistant to Fas-induced apoptosis in the presence of 200 μM YVADcmk, even after a 60 hour incubation (Fig. 1a,b). The addition of YVADcmk inhibited apoptosis in a dose-dependent manner (Fig. 1c,d): in the presence of 50 μM YVADcmk, 50 % of the cells treated with anti-Fas antibody died within 24 hours; in the presence of 300 μM YVADcmk, 100 % of the cells survived. Note that YVADcmk was diluted in DMSO, which is known to act as a differentiating agent for primary cultures of hepatocytes; at the concentrations used here, however, DMSO *per se* conferred no protective effect against Fas-mediated apoptosis (data not shown).

The intraperitoneal or intravenous administration of anti-Fas antibody in mice leads to death [3]. That this results from severe liver damage was shown by the liver-specific expression of a *Bcl-2* transgene, which prevented the lethal effects of the antibody [24]. To evaluate the effect of ICE inhibitor on Fas-mediated apoptosis *in vivo*, mice were treated with YVADcmk (4 μmole) 2 hours before injection of anti-Fas antibody. Most of the mice (93 %) survived and were healthy, whereas all untreated mice died 3–4 hours after antibody injection (Fig. 2a). Histological tissue sections of surviving mice were examined for liver damage and regenerative features. The livers of YVADcmk-treated mice were protected against lethal hepatic cytolysis, but had many areas of hepatocyte injury 12 and 24 hours after antibody injection. This damage consisted of numerous typical apoptotic bodies surrounded by polynuclear granulocyte, lymphocyte and macrophage infiltrates; no such damage was seen in control mice (treated with YVADcmk but not exposed to anti-Fas antibody; data not shown). The anti-apoptotic properties of YVADcmk *in vivo* were confirmed by the TUNEL assay (terminal deoxynucleotidyl transferase (TdT)-mediated dUTPbiotin nick end-labeling), which detects DNA fragmentation — a hallmark of apoptosis. By 1.5 hours after anti-Fas antibody injection, the livers of unprotected mice had numerous TUNEL-positive cells

Figure 1

Effect of YVADcmk on Fas- and TNF-α-mediated hepatocyte cytotoxicity *in vitro*. (a,b) Primary mouse hepatocytes were incubated with anti-Fas antibody in the presence (open bars and open circles) or absence (filled bars and filled circles) of YVAD-cmk. (c,d) Dose-dependent inhibition of Fas-induced apoptosis by YVAcmk. Primary hepatocytes were incubated for 24 h with various concentrations of YVADcmk in the presence (filled bars and filled circles) or absence (open bars and open circles) of anti-Fas antibody. (e,f) Primary hepatocytes were incubated with recombinant murine TNF-α and actinomycin D (ActD) in the presence (open bars and open circles) or absence (closed bars and filled circles) of YVADcmk. Control experiments showed that YVADcmk solvent (DMSO) did not interfere with hepatocyte apoptosis (see text). Percent apoptotic hepatocytes (Hoechst staining) and viability (blue trypan exclusion) were obtained from triplicate experiments.



(Fig. 3a), whereas the extent of the apoptosis was reduced significantly in livers from YVADcmk-protected mice (Fig. 3b). Mitoses appeared in the parenchyma without any obvious zonal distribution within 1 day and persisted at day 4 (Fig. 3c). Progressively, apoptotic bodies disappeared within 6 days and, at day 13, liver architecture was indistinguishable from that of normal liver. Plasmatic transaminase levels increased and then decreased progressively to reach normal values concomitant with the disappearance of the apoptotic process (data not shown). The increase in transaminase levels during this process, a process which is usually described as a nonlytic event, probably results from insufficient phagocytosis [3].

The duration of the YVADcmk protective effect was tested by injecting anti-Fas antibody at day 1, 2 or 3 after administration of YVADcmk. Only the mice injected at day 3 died, suggesting that the effect of YVADcmk was transient. Histological examination of liver, heart, lung, spleen, thymus, lymph nodes, kidney and brain was performed in three mice one month after a single YVADcmk treatment, revealing no apparent cytotoxicity. Finally, the long-term survival of protected mice confirms that blockage of ICE-like proteases, and thereby inhibition of massive hepatic apoptosis, is highly efficient in preventing lethality induced by anti-Fas antibody injection.

TNF-α is a potent mediator of hepatocyte apoptosis and liver failure [4,5]. As the signalling pathway of the TNF type I-receptor requires an ICE-like protease [9,11], we examined whether YVADcmk treatment could protect against TNF-induced hepatocyte apoptosis and liver damage. As shown above for Fas-induced apoptosis, addition of YVADcmk (200 μM) blocked TNF-induced apoptosis of primary hepatocytes, which occurred within 24 hours in the absence of YVADcmk (Fig. 1e,f). YVADcmk protection against TNF-induced liver damage *in vivo* was tested by injection of YVADcmk and TNF-α. As the *in vivo* hepatic apoptosis mediated by TNF-α is known to require transcriptional arrest [4], mice were co-treated with ActD. The high dose (9 μg kg^{-1}) of TNF-α injected into ActD-sensitized mice induced liver damage (Fig. 3e) and death of all the mice in less than 7 hours. Interestingly, YVADcmk administered before TNF-α into ActD-sensitized mice

Figure 2

In vivo protective effect of YVADcmk on Fas-induced mortality. (a) Preventive effect of YVADcmk. Mice were injected intraperitoneally with YVADcmk 2 h before intravenous injection of anti-Fas antibody (open circles). (b) Curative effect of YVAD-cmk. Mice were injected intraperitoneally with YVAD-cmk 2 h after intravenous injection of anti-Fas antibody (open circles). Control mice received anti-Fas antibody alone (filled squares and filled circles) or YVAD-cmk alone (open squares).

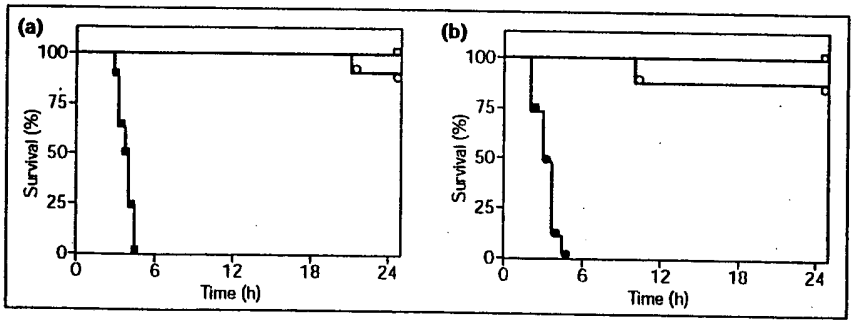
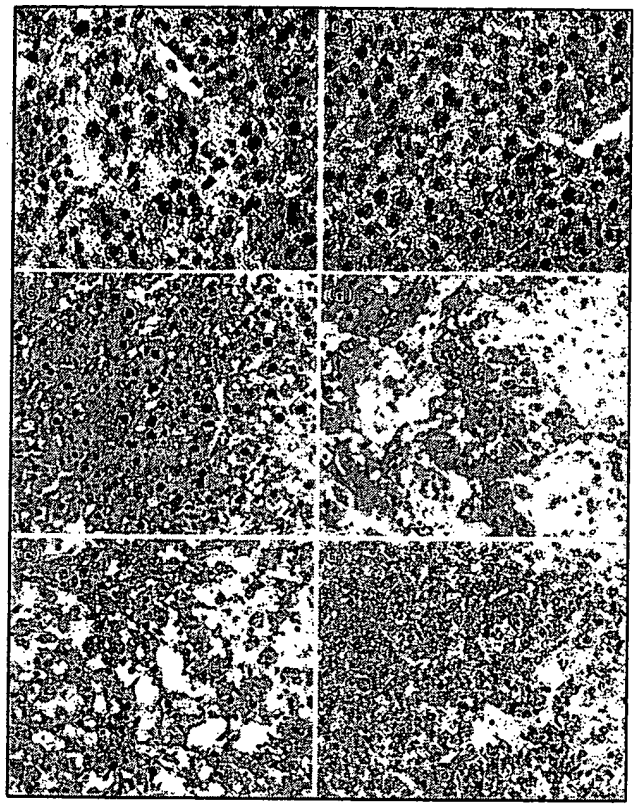


Figure 3



Liver sections from untreated and YVAD-cmk-treated mice. (a,b) TUNEL *in situ* detection of apoptosis, ×250. (a) Liver section from a YVADcmk-untreated mouse 1.5 h after intravenous injection of anti-Fas antibody. Note the presence of multiple stained nuclei (arrows). (b) Liver section from YVAD-cmk-pretreated mouse 1.5 h after intravenous injection of anti-Fas antibody. Rare stained nuclei are detected (arrows). (c,d) Histological analysis, ×200. (c) Liver section from YVAD-cmk-pretreated mouse prepared 4 days after intravenous injection of anti-Fas antibody. The liver has partially recovered its normal organization, despite the presence of mononuclear cell infiltrates and some hepatocyte mitosis. The same histological features were observed with liver sections of rescued mice, which had received YVAD-cmk 2 h after anti-Fas antibody injection. (d) Liver section from untreated mouse prepared 2 h after a single intravenous injection of anti-Fas antibody. The liver exhibits architectural disorganization, numerous apoptotic bodies (arrows) and infiltration of blood cells. (e) Liver section from untreated mice prepared upon death, 7 h after co-injection of ActD and TNF-α. The liver section shows a total disorganization of tissue architecture with numerous apoptotic bodies (arrows). (f) Liver section from YVADcmk-treated mouse was performed 7 h after injection of YVADcmk followed by ActD and TNF-α. The liver displays few scattered apoptotic bodies.

blocked the appearance of liver damage characterized by apoptosis (Fig. 3f) and prevented the death of animals. Considering the wide range of cells sensitive to TNF cytotoxicity [25], the causal relationship between TNF-induced death and TNF-induced liver damage has not yet been demonstrated formally. The ability of YVADcmk to protect against TNF-lethal cytotoxicity could thus be related to its protection of other cell types *in vivo*. Note that inflammatory response syndrome observed in septic shock is mainly related to TNF-α production [26–28] and is attenuated in ICE-deficient mice [29]. It will be thus of great interest to assess the ability of YVADcmk to abolish multiple organ failure in septic shock experimental models.

To evaluate the curative potential of YVADcmk treatment against Fas-induced liver damage, we tested its ability to stop an ongoing lethal hepatic cytolysis. Time-course analysis of hepatic histological damage induced by anti-Fas antibody administration showed that 50–70 % of the liver was destroyed within 2 hours (Fig. 3d). As this experimental damage could be relevant to clinical lesions observed during liver failure [3], YVADcmk was administrated 2 hours after anti-Fas antibody injection. Under these conditions, 86 % of the mice were rescued, whereas all animals died in the control group, untreated with YVADcmk (Fig. 2b). It is noteworthy that YVADcmk treatment is rapidly effective, considering the short survival of mice treated with anti-Fas antibody (3–4 hours). Biochemical analysis of hepatic transaminases and liver histology of rescued mice showed apoptosis followed by a regenerative process similar to that described for protected mice in the preventive protocol (data not shown). These data demonstrate that YVADcmk is able to overcome the rapid progression of dramatic hepatic damage induced by Fas activation.

Our results suggest that *in vivo* inhibition of ICE-dependent apoptosis mediated by Fas or TNF-α represents an attractive approach for treating liver injuries, including including those caused by inflammatory, viral and autoimmune diseases [1]. As we can assume that, during fulminant hepatitis, multiple signals account for massive hepatic destruction, it would be of interest to assess ICE-mediated protection in other models. This hypothesis is supported by our preliminary experiments demonstrating an *in vivo* protective effect of YVADcmk against LPS-induced fulminant hepatitis (N.R. and A. Mignon, unpublished data). Recently, transgenic liver-specific expression of Bcl-2 has proven its efficacy in protecting mice against Fas-induced lethality [24]. However, therapeutic application of Bcl-2 will encounter the difficulties inherent to gene-transfer approaches. Our results provide evidence that a simple galenic formulation of ICE-inhibiting drugs has a curative effect on Fas-mediated liver apoptosis. This opens the field for new treatments of viral fulminant hepatitis.

Materials and methods

In vitro *experiments*

Hepatocytes were isolated by *in situ* collagenase perfusion [23]. The perfused liver was minced, cells were suspended in M199 medium with 10 % fetal calf serum (FCS; Gibco-BRL) and filtered through a 70 μm mesh filter. Viability was over 90 % according to trypan blue exclusion. Cells were counted and plated at a density of 5×10^5 cells per 35 mm dishes in M199 medium containing 10 % FCS. The cells were allowed to adhere for 3 h; the medium was then removed and replaced by M199 containing 10^{-8} M insulin, 10^{-6} M thyroid hormone and 10^{-6} M dexamethasone. The *ex vivo*

protective effect of YVADcmk (Ac-Tyr-Val-Ala-Asp-chloromethylketone) (Bachem Biochimie SARL, Bale) was explored by culturing primary hepatocytes in the presence or absence of 200 μM YVADcmk in dimethyl sulfoxide (DMSO). At the same time anti-Fas antibody (1 μg ml^{-1}) (Pharmingen), supplemented with 10 μg ml^{-1} CHX or recombinant murine TNF-α (20 ng ml^{-1}) (Genzyme) supplemented with ActD (333 nM) (Sigma) were added. Cells were monitored at times up to 60 h and the percentage of viable cells assessed by trypan blue exclusion. Approximately 200 cells were counted in each sample. To monitor apoptosis, Hoechst 33258 staining was performed as described [23].

In vivo *experiments*

To explore the *in vivo* preventive effect of YVADcmk, a group of 14 mice were injected intraperitoneally with YVADcmk (4 μmole in 200 μl PBS) 2 h prior to intravenous injection of anti-Fas antibody (10 μg in 100 μl 0.9 % NaCl) or prior to intravenous injection of 9 μg kg^{-1} of TNF-α and intraperitoneal administration of ActD (16 μg in 200 μl PBS). To explore the *in vivo* curative effect of YVADcmk, a group of 7 mice were injected intraperitoneally with YVADcmk (4 μmole in 200 μl PBS) 2 h after intravenous injection of anti-Fas antibody (10 μg in 100 μl 0.9 % NaCl). As controls, 15 mice received anti-Fas antibody alone (10 μg in 100 μl 0.9 % NaCl) and 3 mice received YVADcmk alone (4 μmole in 200 μl PBS). 8-week-old B6D2/F1 mice were used in all experiments.

Histological examinations, TUNEL and serum analysis

Livers were excised and immediately transferred to Bouin's and formalin-acetic acid–alcohol fixatives. Samples embedded in Paraplast were cut at 5 μm and stained with hematoxylin and eosin. Using light microscopy, 3 fields of each sample were analyzed for typical apoptotic features: marked condensation of chromatin, cell shrinkage and apoptotic bodies. Apoptosis severity was evaluated by a ratio of the number of apoptotic hepatocytes to the total hepatocytes of the field. The TUNEL procedure was performed with the '*In situ* cell death detection kit, POD' (Boehringer Mannheim). For serum analysis, biochemical parameters of the serum (ALT, alanine aminotransferase, and AST, aspartate aminotransferase) were quantified using a standard clinical automatic analyzer (Hitachi, Type 7150).

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